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Il Farmaco 58 (2003) 439–444
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# Pyrimidin-8-on[2,1-f]theophylline-9-alkylcarboxylic acids amides as $A_1$ and $A_{2A}$ adenosine receptor ligands

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Received 21 September 2002; accepted 15 February 2003

**Abstract**

Starting from the appropriate esters (**1**–**3**), pyrimidin-8-on[2,1-f]theophylline-9-alkylcarboxylic acids amides (**4**–**10**) were synthesized and evaluated as hydrochlorides (**4a**–**10a**) for their affinity at brain $A_1$ and $A_{2A}$ adenosine receptor subtypes. Radioligand binding assay showed that morpholine-ethyl(-propyl) amide of pyrimidin-8-on[2,1-f]theophylline-9-acetic acid (**4a**, **5a**) exhibited greater affinity and selectivity for $A_1$ and $A_{2A}$ receptors than parent compounds (theophylline and caffeine), with $K_i$ values: 12.2 and 3.1 μM for $A_1$ and 1.11 and 5.89 μM for $A_{2A}$, respectively. Morpholine-ethyl amide of pyrimidin-8-on[2,1-f]theophylline-9-propanoic acid (**6a**) and the dimethyl-amino analog (**10a**) exhibited much lower affinity for $A_1$ and $A_{2A}$ adenosine receptors, with $K_i$ values, respectively: 53.9 and 72.6 μM for $A_1$ and 120 and 115 μM for $A_{2A}$. Morpholine-propyl amide of pyrimidin-8-on[2,1-f]theophylline-9-propanoic acid (**7a**) exhibited relatively higher affinity for $A_1$ adenosine receptor with $K_i$ value 32.8 μM, comparable to caffeine, but it showed weaker affinity to $A_{2A}$ receptor. The variation of affinity at $A_1$ and $A_{2A}$ adenosine receptors depends on the structure of substituent in N9-position of fused tricyclic theophylline derivatives. The most interesting were morpholino-ethyl(-propyl) amides of pyrimidin-8-on[2,1-f]theophylline-9-acetic acid (**4a**, **5a**). The longer alkylene chain (propylene) between amide nitrogen and the basic center (**5a**) resulted in higher $A_1$ but lower $A_{2A}$ receptor affinity.


*Keywords:* Pyrimido[2,1-f]theophyllines; Alkylcarboxylic acids amides; $A_1$ and $A_{2A}$ adenosine receptor ligands; Tricyclic theophylline derivatives

## 1. Introduction

Highly selective agonists and antagonists have been designed for $A_1$ and $A_{2A}$ adenosine receptors. Those agents are potentially useful in the treatment of cardiovascular, renal and central nervous system disorders [1]. Methylxanthines, including theophylline and caffeine, are classical adenosine receptor antagonists [1,2], with limited selectivity for $A_1$ and $A_2$ subtypes. The $K_i$ values are 14 and 19 μM for theophylline and 41 and 43 μM for caffeine, respectively. It has been found that the presence of a substituent at 1 and 8-position of xanthine is important for high adenosine receptor affinity, while the substitution at 3-position is not crucial [2]. The first selective $A_{2A}$ antagonist with high affinity was 3,7-dimethyl-1-propargyl-xanthine (DMPX) [3]. Some potent and selective $A_{2A}$ adenosine receptor antagonists in the group of 1-propargyl-8-styrylxanthine with polar substituent in aromatic ring were also developed [4,5]. Configurationally stable analogs of 8-styrylxanthine exhibited high affinity for $A_{2A}$ receptor with moderate selectivity [6]. In the study on tricyclic theophylline derivatives (Fig. 1) we have found that imidazol, pyrimidine or diazepine ring fused in the position 7,8 of theophylline change the profile of CNS activity in comparison to the reference compound (theophylline) to sedative, hypothermizing and neuroleptic-like properties [7–11].

Tricyclic theophylline derivatives, with various -aryl (-heteroaryl) substituted piperazine evaluated for their affinity for 5-$HT_{1A}$ and 5-$HT_{2A}$ serotonine receptors were classified as postsynaptic antagonists or partial agonists of 5-$HT_{1A}$ subtype [12]. Some of above

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doi:10.1016/S0014-827X(03)00066-1

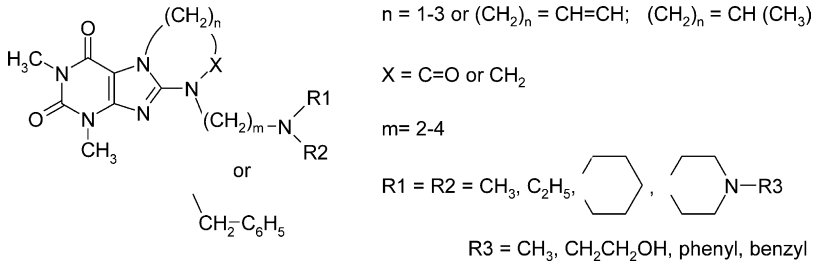



Fig. 1. Structure of pharmacologically investigated imidazo-, pyrimido- and diazepino-[2,1-f]theophyllines.

described tricyclic derivatives in the class of pyrimido[2,1-f]theophyllines with basic dialkylamino-alkyl substituent in the third ring (Fig. 1), exhibited the affinity for adenosine receptors in the same range as theophylline and caffeine [13]. A piperazine ring with the small substituent (R3) on the nitrogen, such as methyl or 2-hydroxyethyl, was favorable for relatively high affinity of the compounds, however the presence of a large substituent such as phenyl or benzyl reduced the affinity. The introduction of an oxo group into the third ring (lactam structure) lead to $A_1$ selective compounds [13]. Dialkylaminopropyl and piperazinopropyl substituted analogs of pyrimido- and pyrimidin-8-on[2,1-f]theophylline were also investigated in the aspect of their agonistic versus antagonistic properties for adenosine receptors. The study of the influence of GTP and its stable analog, on binding of the compounds to the receptor, allow to assume that the tricyclic theophylline derivatives are adenosine receptor antagonists rather than agonists [13].

In the present study, we have synthesized and tested for $A_1$ and $A_{2A}$ adenosine receptor affinity novel pyrimidin-8-on[2,1-f]theophylline derivatives with dialkylamino-alkylamide of acetic, propanoic or butanoic acid rest substituted in the position N9. As a reference compounds theophylline and caffeine were used.

## 2. Investigations, results and discussion

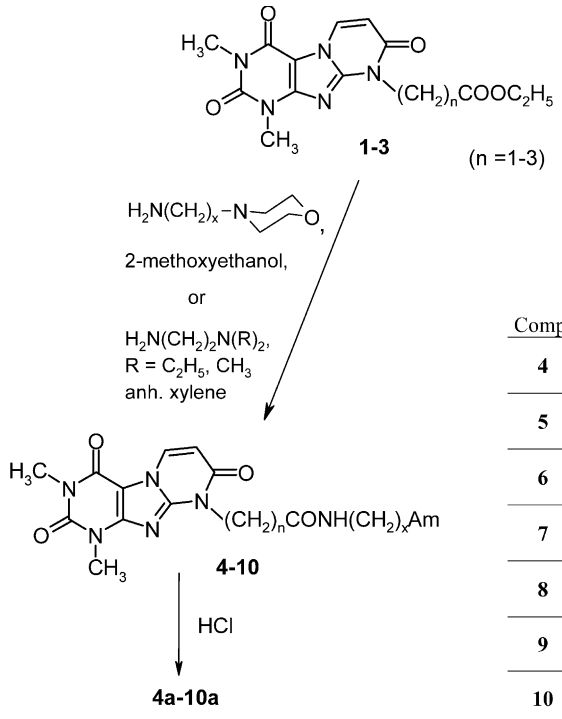
### 2.1. Chemistry

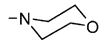
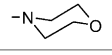
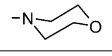
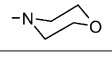
The synthesis was performed by alkylation of N9-unsubstituted pyrimidin-8-on[2,1-f]theophylline [14] with ethylchloroacetate (**1**), ethyl acrylate (**2**) and ethyl 4-bromobutyrate (**3**) [15]. In the reaction of esters **1**–**3** with morpholine-ethyl(-propyl)-amine in boiling 2-methoxyethanol the appropriate morpholino-alkylamides **4**–**8** were obtained. The dialkylamino-alkylamides **9**, **10** were synthesized by condensation of the esters **1** and **2** with dialkylamino-ethylamines in boiling anhydrous xylene. The final amides **4**–**10** were transformed into water soluble hydrochlorides **4a**–**10a** by saturating the suspensions of the bases **4**–**10** with HCl gas in anhydrous ethanol (Scheme 1).

### 2.2. Pharmacology

Xanthine derivatives, including theophylline and caffeine, act through the blockade of extracellular adenosine receptors. The new tricyclic compounds **4a**–**10a** (Scheme 1) were tested for their affinity at brain $A_1$ and $A_{2A}$ receptor subtypes. The results from radioligand binding assays are summarized in Table 1.

Compounds **4a** and **5a** are potent $A_1$ and $A_{2A}$ receptor ligands with $K_i$ within the value 12.2 and 3.1 μM for $A_1$ receptor affinity and 1.1 and 5.9 μM for $A_{2A}$. We have found that compound **4a** showed affinity for $A_1$ receptor at the same range as theophylline, and it demonstrated more than threefold higher affinity for $A_1$ receptor than caffeine, while the affinity of **4a** for $A_{2A}$ receptor was significantly higher, almost 20-fold than theophylline, and 40-fold higher than caffeine. Significant $A_1/A_2$ selectivity for compound **4a** was also observed. Compound **5a** exhibited $A_1$ receptor affinity, almost fivefold higher than theophylline and 14-fold higher than caffeine. It has been shown that $A_{2A}$ receptor affinity of **5a** was threefold higher than theophylline, and more than sevenfold higher than caffeine with relatively higher selectivity for $A_1$ receptor. On the other hand, compounds **6a** and **10a** exhibited lower affinity for $A_1$ and much lower affinity for $A_{2A}$ receptor, which ranged from 54 to 73 μM for $A_1$ and 115 to 120 μM for $A_{2A}$ receptor. The selectivity towards $A_1$ receptor found for compounds **6a** and **10a** was quite significant. Compound **7a** showed relatively lower affinity for $A_1$ receptor ($K_i = 32$ μM), compared with theophylline and slightly higher than caffeine. Very weak $A_{2A}$ receptor affinity of compound **7a** was observed; the $A_1/A_2$ selectivity was quite considerable. Morpholine-ethyl amide (**8a**) and diethyl-amino-ethyl amide (**9a**) were not tested at concentration higher than 10 μM. The inhibition of $A_1$ and $A_{2A}$ receptor affinity after administration of compound **8a** was 1–26%, whereas compound **9a** showed 41–42% inhibition of $A_1$ and $A_{2A}$ receptor affinity in the concentration used.




| Comp. | n | x | Am |
|---|---|---|---|
| **4** | 1 | 2 |  |
| **5** | 1 | 3 |  |
| **6** | 2 | 2 |  |
| **7** | 2 | 3 |  |
| **8** | 3 | 2 |  |
| **9** | 1 | 2 | $-N(C_2H_5)_2$ |
| **10** | 2 | 2 | $-N(CH_3)_2$ |

Scheme 1.

A few remarks on the structure–affinity relationships may be drawn on the basis of adenosine receptor binding data:

- the affinity depends on the length of alkyl chain in alkylcarboxylic moiety and also on the structure of the amide fragment.
- acetic acid moiety substituted in the 9 position (**4a**, **5a**) seems to be optimal for affinity to $A_1$ and $A_{2A}$ adenosine receptors. Higher homologues (**6a**–**8a**) presented moderate to low affinity with preference for $A_1$ receptor subtype.
- elongation of the alkylene chain between amide nitrogen and the basic center plays an important role in $A_1$ and $A_{2A}$ adenosine receptor affinity. Two-carbon chain spacer present in the structure of compound **4a** resulted in higher affinity to $A_{2A}$ while its higher homologue (**5a**) showed preference for $A_1$ receptor.
- the presence of morpholine with high lipophylic cyclic ether structure (**4a**–**8a**) in most cases (with the exception of butanoic acid derivative **8a**) revealed $A_1$ and $A_{2A}$ receptor affinity.

The new water soluble morpholino-ethyl-(propyl-)amides of pyrimidin-8-on[2,1-f]theophylline-9-acetic acid (**4a**, **5a**), with improved affinity for $A_1$ receptors, seem to be an interesting subject for further chemical modifica-

Table 1
Affinities of amides **4a**–**10a** and related compounds to rat brain $A_1$ and $A_{2A}$ adenosine receptors

| Comp. | $A_1$ adenosine receptor (rat brain cortical membranes) [$^3$H]CCPA ($n = 3$) | | $A_{2A}$ adenosine receptor (rat brain striatal membranes) [$^3$H]MSX-2 ($n = 3$) | | $A_1$ selectivity ($A_{2A}/A_1$) |
|---|---|---|---|---|---|
| | Inhibition (%) ($c = 10$ μM) | $K_i \pm$SEM (μM) | Inhibition (%) ($c = 10$ μM) | $K_i \pm$SEM (μM) | |
| Theophylline | | 14±1 | | 19±2 | 1.36 |
| Caffeine | | 41±6 | | 43±9 | 1.05 |
| **4a** | 24±4 | 12.2±0.00 | 79±6 | 1.11±0.53 | 0.090 |
| **5a** | 21±4 | 3.11±0.08 | 53±6 | 5.89±0.52 | 1.89 |
| **6a** | 5±1 | 53.9±1.8 | 0±0 | 120±15 | 2.23 |
| **7a** | 0±0 | 32.8±7.0 | 5±2 | 235±5 | 7.16 |
| **8a** | 1±1 | n.d. | 26±4 | n.d. | |
| **9a** | 42±2 | n.d. | 41±3 | n.d. | |
| **10a** | 2±2 | 72.6±9.1 | 8±4 | 115±26 | 1.58 |

n.d. = not determined.

tion directing to active and more selective $A_1$ and $A_2$ receptor ligands.

## 3. Experimental

### 3.1. Chemistry

Melting points are given uncorrected. The UV spectra were obtained on UV–Vis spectrophotometer Perkin–Elmer Lambda 12 (conc. $1 \times 10^{-5}$ mol/dm$^3$ in MeOH). $^1$H NMR Spectra of all synthesized bases were recorded on Varian 200 BB (300 MHz) instrument with TMS as internal standard in $CDCl_3$ (chemical shifts in $\delta$, ppm). MS spectra of selected compounds were performed on mass spectrometer type AMD 604 with direct inlet (ionization energy 70 eV). The purity of the products were confirmed by TLC on Merck plates (Kieselgel 60 $F_{254}$), the respective solvents were used and the spots were visualized under UV light. The new derivatives were purified by recrystallization and analyzed by quantitative elemental (C, H, N) analysis. The results were within $\pm$0.4% of the theoretical values (data not shown) (Table 2).

### 3.2. Chemical procedures and analytical data

#### 3.2.1. Morpholino-alkyl-amide of pyrimidin-8-on[2,1-f]theophylline-9-acetic (-propanoic, -butanoic) acid (4–8)

*3.2.1.1. General procedure.* A mixture of the appropriate esters **1**–**3** (0.005 mol), amino-ethyl(-propyl)-morpholine (0.01 mol) and 2-methoxyethanol (15 ml) was refluxed for 10 h. Then the reaction mixture was cooled, the precipitated solid product was filtered off, washed with water and then purified by recrystallization from 96° EtOH.

*3.2.1.2. Morpholinoethylamide of pyrimidin-8-on[2,1-f]theophylline-9-acetic acid (**4**).* $^1$H NMR $\delta = 2.51$–2.58 (m, 6H, $CH_2N(CH_2)_2$); 3.46–3.57 (m, 2H, $NHCH_2$); 3.42 (s, 3H, N3$CH_3$); 3.68 (s, 3H, N1$CH_3$); 3.71–3.74 (t, 4H, $(CH_2)_2O$); 4.92 (s, 2H, N9$CH_2$); 6.31–6.34 (d, 1H, C7*H*, $J = 7.4$ Hz); 6.77 (brs, 1H, N*H*); 8.54–8.57 (d, 1H, C6*H*, $J = 7.4$ Hz). UV: $\lambda_{max1}$ (log $\varepsilon$) = 249 nm (4.84); $\lambda_{max2}$ (log $\varepsilon$) = 289 nm (4.15).

*3.2.1.3. Morpholinopropylamide of pyrimidin-8-on[2,1-f]theophylline-9-acetic acid (**5**).* $^1$H NMR $\delta = 1.78$–1.86 (m, 2H, $CH_2CH_2CH_2$); 2.51–2.59 (m, 6H, $CH_2N(CH_2)_2$); 3.39–3.43 (m, 2H, $NHCH_2$); 3.44 (s, 3H, N3$CH_3$); 3.62 (s, 3H, N1$CH_3$); 3.73–3.79 (t, 4H, $(CH_2)_2O$); 4.96 (s, 2H, N9$CH_2$); 6.30–6.33 (d, 1H, C7*H*, $J = 7.9$ Hz); 7.41 (brs, 1H, N*H*); 8.58–8.60 (d, 1H, C6*H*, $J = 7.6$ Hz). UV: $\lambda_{max1}$ (log $\varepsilon$) = 250 nm (5.00); $\lambda_{max2}$ (log $\varepsilon$) = 284 nm (4.75).

*3.2.1.4. Morpholinoethylamide of pyrimidin-8-on[2,1-f]theophylline-9-propanoic acid (**6**).* $^1$H NMR $\delta =$ 2.46–2.52 (m, 6H, $CH_2N(CH_2)_2$); 2.73–2.80 (t, 2H, $CH_2CO$); 3,33–3,41 (m, 2H, $NHCH_2$); 3.43 (s, 3H, N3$CH_3$); 3.62 (s, 3H, N1$CH_3$); 3.69–3.74 (t, 4H, $(CH_2)_2O$); 4.56–4.64 (t, 2H, N9$CH_2$); 6.22 (brs, 1H, N*H*); 6.28–6.31 (d, 1H,C7*H*, $J = 7.8$ Hz); 8.51–8.55 (d, 1H, C6*H*, $J = 7.7$ Hz). MS $m/z = 431$ (1) $M^+$, 388(2), 368(1), 344(3), 319(1), 313(1), 248(5), 247(5), 129 (3), 100(100), 98(7), 69(6), 57(9), 40(24). UV: $\lambda_{max1}$ (log $\varepsilon$) = 251 nm (4.64); $\lambda_{max2}$ (log $\varepsilon$) = 285 nm (4.56).

*3.2.1.5. Morpholinopropylamide of pyrimidin-8-on[2,1-f]theophylline-9-propanoic acid (**7**).* $^1$H NMR $\delta =$ 1.67–1.73 (m, 2H, $CH_2CH_2CH_2$); 2.46–2.51 (m, 6H,

Table 2
Yields and analytical data of compounds **4**–**10a**

| Comp. | M.p. (°C) | Yield (%) | Molecular formula (molecular weight) | TLC (solvent) |
|---|---|---|---|---|
| **4** | 213–215 | 48 | $C_{18}H_{23}N_7O_5$ (417.41) | 0.29 (A), 0.87(B) |
| **4a** | 273–275 | | $C_{18}H_{23}N_7O_5HCl$ (453.88) | |
| **5** | 183–185 | 46 | $C_{19}H_{25}N_7O_5$ (431.45) | 0.31(A), 0.80(B) |
| **5a** | 260–262 | | $C_{19}H_{25}N_7O_6HCl$ (467.94) | |
| **6** | 253–255 | 53 | $C_{19}H_{25}N_7O_5$ (431.45) | 0.27(A), 0.85(B) |
| **6a** | 208–210 (decomp.) | | $C_{19}H_{25}N_7O_5HCl$ (467.94) | |
| **7** | 210–212 | 51 | $C_{20}H_{27}N_7O_5$ (445.47) | 0.31(A), 0.81(B) |
| **7a** | 182–184 (decomp.) | | $C_{20}H_{27}N_7O_5HCl$ (481.97) | |
| **8** | 261–263 | 45 | $C_{20}H_{27}N_7O_5$ (445.47) | 0.28(A), 0.76(B) |
| **8a** | 268–270 | | $C_{20}H_{27}N_7O_5HCl$ (481.97) | |
| **9** | 216–218 | 52 | $C_{18}H_{25}N_7O_4$ (403.44) | 0.45(A), 0.87(B) |
| **9a** | 246–248 | | $C_{18}H_{25}N_7O_4HCl$ (439.94) | |
| **10** | 222–224 | 56 | $C_{17}H_{23}N_7O_4$ (389.41) | 0.33(A), 0.80(B) |
| **10a** | 164–166 (decomp.) | | $C_{17}H_{23}N_7O_4HCl$ (425.51) | |

Solvent system: (A) benzene:acetone (7:3); (B) benzene: acetone:methanol (1:1:1).

$CH_2N(CH_2)_2$); 2.73–2.80 (t, 2H, $CH_2CO$); 3.33–3.36 (m, 2H, $NHCH_2$); 3.41 (s, 3H, $N3CH_3$); 3.59 (s, 3H, $N1CH_3$); 3.69–3.73 (t, 4H, $(CH_2)_2O$); 4.53–4.57 (t, 2H, $N9CH_2$); 6.25–6.31 (d, 1H, C7*H*, $J = 7.7$ Hz); 7.27 (brs, 1H, N*H*); 8.48–8.52 (d, 1H, C6*H*, $J = 7.7$ Hz). MS *m*/*z* = 445 (2) $M^+$, 414(10), 402(15), 359(2), 346(1), 318(2), 302(6), 274(5), 248 (20), 247(40), 218(3), 190(4), 167(3), 162(8), 135(5), 100(100), 86(7), 70(6), 56(10), 40(8). UV: $\lambda_{max1}$ (log $\varepsilon$) = 248 nm (4.76); $\lambda_{max2}$ (log $\varepsilon$) = 286 nm (4.71).

*3.2.1.6. Morpholinoethylamide of pyrimidin-8-on[2,1-f]theophylline-9-butanoic acid (**8**).* $^1$H NMR $\delta = 1.65$–1.73 (m, 2H, $CH_2CH_2CH_2$); 2.45–2.50 (m, 6H, $CH_2N(CH_2)_2$); 2.68–2.73 (t, 2H, $CH_2CO$); 3.32–3.38 (m, 2H, $NHCH_2$); 3.42 (s, 3H, $N3CH_3$); 3.60 (s, 3H, $N1CH_3$); 3.66–3.72 (t, 4H, $(CH_2)_2O$); 4.55–4.60 (t, 2H, $N9CH_2$); 6.27–6.29 (d, 1H, C7*H*, $J = 7.7$ Hz); 7.18 (brs, 1H, N*H*); 8.50–8.53 (d, 1H, C6*H*, $J = 7.7$ Hz). UV: $\lambda_{max1}$ (log $\varepsilon$) = 253 nm (4.97); $\lambda_{max2}$ (log $\varepsilon$) = 283 nm (4.86).

*3.2.2. N,N-Diethylaminoethylamide of pyrimidin-8-on[2,1-f]theophylline-9-acetic acid (**9**)*

The mixture of the ester **1** (1.67 g; 0.005 mol), *N*,*N*-diethylaminoethylamine (1.18 g; 0.01 mol) and anhydrous xylene (15 ml) were refluxed for 15 h. After cooling the precipitated product was filtered off, washed with water and then purified by recrystallization from 96° EtOH.

$^1$H NMR $\delta = 1.00$–1.06 (t, 6H, $N(CH_2CH_3)_2$); 2.53–2.62 (m, 6H, $CH_2N(CH_2)_2$); 3.27–3.37 (m, 2H, $NHCH_2$); 3.40 (s, 3H, $N3CH_3$); 3.58 (s, 3H, $N1CH_3$); 4.60 (s, 2H, $N9CH_2$); 6.25–6.28 (d, 1H, C7*H*, $J = 7.8$ Hz); 6.70 (brs, 1H, N*H*); 8.48–8.50 (d, 1H, C6*H*, $J = 7.7$ Hz). UV: $\lambda_{max1}$ (log $\varepsilon$) = 255 nm (4.73); $\lambda_{max2}$ (log $\varepsilon$) = 286 nm (4.65).

*3.2.3. N,N-Dimethylaminoethylamide of pyrimidin-8-on[2,1-f]theophylline-9-propanoic acid (**10**)*

The mixture of the ester **2** (1.74 g; 0.005 mol), *N*,*N*-dimethylaminoethylamine (0.88 g; 0.01 mol) and anhydrous xylene (15 ml) were refluxed for 15 h. After cooling the precipitated product was filtered off, washed with water and then purified by recrystallization from 96° EtOH.

$^1$H NMR $\delta = 2.29$ (s, 6H, $N(CH_3)_2$); 2.45–2.51 (t, 2H, $CH_2N(CH_3)_2$); 2.71–2.79 (t, 2H, $CH_2CO$); 3.30–3.39 (m, 2H, $NHCH_2$); 3.43 (s, 3H, $N3CH_3$); 3.60 (s, 3H, $N1CH_3$); 4.55–4.62 (t, 2H, $N9CH_2$); 6.26–6.30 (d, 1H, C7*H*, $J = 7.8$ Hz); 6.57 (brs, 1H, N*H*); 8.48–8.52 (d, 1H, C6*H*, $J = 7.7$ Hz). UV: $\lambda_{max1}$ (log $\varepsilon$) = 251 nm (4.54); $\lambda_{max2}$ (log $\varepsilon$) = 286 nm (4.46).

*3.2.4. Hydrochlorides of **4**–**10** (**4a**–**10a**)*

The suspension of the base **4**–**10** (1.0 g) in 20 ml of anhydrous EtOH was saturated with HCl gas while cooling on an ice-water bath. The precipitate was gradually dissolved and the salt precipitation was observed. The mixture was frozen at −20 °C for 12 h, the precipitate was collected by filtration and recrystallized from anhydrous EtOH.

*3.3. Pharmacology*

The compounds were investigated in radioligand binding assays at $A_1$ adenosine receptor of rat brain cortical membranes using the $A_1$ selective radioligand [$^3$H]2-chloro-N6-cyclopentyladenosine ([$^3$H]CCPA), and at $A_{2A}$ adenosine receptor of rat striatal membranes using the $A_{2A}$ selective radioligand [$^3$H]3-(3-hydroxypropyl)-7-methyl-8-(*m*-methoxystyryl)-1-propargyl xanthine ([$^3$H]MSX-2).

Inhibition of binding of [$^3$H]CCPA to $A_1$ adenosine receptor of rat cerebral cortical membranes and the inhibition of [$^3$H]MSX-2 to $A_{2A}$ receptor of rat striatal membranes were determined as described previously by Klotz et al. [16] and Müller et al. [17]. Frozen rat brains were obtained from Pel Freez®, Rogers, Arkansas, USA, and thawed at 4 °C. Frontal cortex was dissected as $A_1$ receptor source, right and left striata were dissected for $A_{2A}$ adenosine receptor studies. Tissues were homogenized immediately in 50 mM Tris–HCl buffer (pH 7.4). Membrane fractions were purified by a series of centrifugation steps according to a modified method of Lowry [18], for cortical membranes, and according to Bruns et al. [19], for striatal membranes. Final protein pellets were re-suspended in 50 mM Tris–HCl buffer pH 7.4 and stored in aliquots (up to a concentration of 4 mg/ml) at −80 °C until used. Protein concentration was determined by the method of Bradford [20], using a Biorad assay kit. Before determination, protein was washed in HEPES–NaOH buffer 10 mM, pH 7.4 to prevent interactions of buffer and the reagents used for the colorimetric analysis of the protein content. Bovine serum albumin was used as a reference standard. About 70 μg/ml protein were used in the assays. Membranes were preincubated with 0.2 IU/ml of adenosine deaminase in order to remove endogenous adenosine. The tested compounds were dissolved in water. Inhibition curves were determined using six to seven different concentrations of the tested compounds spanning three orders of magnitude. At least three separate experiments were performed each in triplicate. Radioligand binding to rat brain cortical membranes was carried out in Tris–HCl buffer (50 mM, pH 7.4). Samples were incubated in a shaking water-bath at 23 °C for 90 min. Nonspecific binding was defined using 10 μM of 2-chloroadenosine and amounted to less than 5% of total binding. [$^3$H]CCPA was used in a final

concentration of 0.5 nM. Protein (ca. 70 μg per tube containing a final volume of 1 ml) was added to start the reaction. Incubation was terminated by rapid filtration using a Brandel 48-channel cell harvester (Brandel, Gaithersburg, MD) through Whatman GF/B glass fiber filters, which had been presoaked in rinse buffer (Tris–HCl 50 mM, pH 7.4). Filters were rinsed three times with 2 ml of Tris–HCl buffer.

Radioligand binding to rat brain striatal membranes was carried out in Tris–HCl buffer. Samples were incubated on a shaking water-bath at 23 °C for 30 min. Nonspecific binding was defined using 50 μM NECA and amounted to less than 25% of total binding. Total binding was determined in the presence of [$^3$H]MSX-2 in a concentration of 1 nM, 70 μg of protein per tube (containing a final volume of 1 ml) was added to start the reaction. Termination of the incubation was performed by rapid filtration through GF/B glass fiber filters, presoaked in 0.5% aqueous polyethylenimine solution for 45 min using a Brandel 48-channel cell harvester. Filters were washed three times with ice-cold Tris–HCl buffer.

Radioactivity of the punched-out wet filters was counted after 9 h of pre incubation with 3 ml of Ultima Gold scintillation cocktail (Canberra Packard, Dreieich, Germany) in a TRICARB 2100TR liquid scintillation counter (Canberra Packard, Dreieich, Germany). The data was analyzed using PRISM Version 3.0 (Graphpad, San Diego, CA); the Cheng Prusoff [21] equation and $K_D$ values of 0.5 nM for [$^3$H]CCPA and 8 nM for [$^3$H]MSX-2 were used to calculate $K_i$ values from $IC_{50}$ values.

## Acknowledgements

This study was partly supported by the Libyan Minister of Education.

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